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Cloning, purification and biochemical properties of a thermostable pectinase from *Bacillus halodurans* M29

Yanzhen Mei[a,*], Yuru Chen[a,b], Ruying Zhai[a], Yang Liu[a]

[a] *Jiangsu Engineering and Technology Research Centre for Microbiology Resource, Jiangsu Key Laboratory for Biodiversity and Biotechnology, Jiangsu Key Laboratory for Microbes and Functional Genomic, College of Life Science, Nanjing Normal University, Nanjing 210023, China*
[b] *School of Biotechnology and Chemical Engineering, Taizhou College, Nanjing Normal University, Taizhou 225300, China*

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ABSTRACT

An M29 strain that can grow under highly alkaline conditions from 40 °C to 65 °C was isolated and identified as *Bacillus halodurans*. The isolate was a Gram-positive, spore-forming, aerobic, and alkaliphilic bacterium. A pectinase was cloned from M29 and expressed in *Escherichia coli* JM109 (DE3). A 39 kDa protein with pectinase activity was purified by heat treatment and with DEAE-Sepharose Fast Flow from culture supernatant to gel electrophoretic homogeneity. Optimal activity was achieved at pH 10 and 80 °C. The purified enzyme was stable from pH 9.5 to 10.5 and had a 1 h half-life at 80 °C. Kinetic experiments at 80 °C with polygalacturonic acid as substrate revealed K_m and V_{max} values of 4.1 g L^{-1} and 351 U mg^{-1} protein, respectively. The pectinase from *B. halodurans* showed high thermostability and may be a valuable candidate enzyme in bioscouring.

1. Introduction

Pectin, a major constituent of cereals, vegetables, fruits, and fibers, is a complex, high-molecular weight, heterogeneous, and acidic-structured polysaccharide. This complex carbohydrate is degraded by pectinolytic microorganisms producing an extracellular enzymatic complex consisting of exopectinases, endopectinases, pectate lyase, and methylesterase [1]. Most of the known pectinolytic microorganisms grow optimally at acidic and neutral pH. Although evidence for pectinolysis at high pH and high thermostable are scarce, this process is potentially useful for application in the fabric industry, for the biopreparation of cotton fabrics and enzymatic polishing of cotton blended fabrics [2–4], and in the paper industry to remedy the retention problems in mechanical pulp bleaching [5]. Conventional methods used in the processing of fibers, such as mechanical processing and treatment of fiber with 12–20% NaOH along with wetting and reducing agents, are inefficient because the gummy material of a fiber is not completely removed and the use of alkali results in environmental pollution. Hence, fiber treatment with alkaline solution containing pectinases is preferred [6]. Several studies have focused on alkaline pectinase. However, the industrial application of most pectate lyases reported in the literature is limited by their thermostability and short half-lives. The alkaline pectinase producers are mainly nonthermo-tolerant aerobes such as various *Bacillus* [7,8], *Xanthomonas* [9], *Aspergillus* [10], *Alkaliflexus* [11], *Natronoflexus* [12], and *Natranaerovirga* [13]. The optimal growth temperature for most of the strains is under 40 °C, and the optimal temperature for pectinase production is below 60 °C. However, low thermostability and half-life are important restrictive factors for practical application. Thus, novel strains with efficient and thermostable alkaline pectinase are continuously explored.

In this study, a thermo-tolerant bacterium belonging to the genus *Bacillus* was newly isolated from soil, China. The bacterium, identified as *Bacillus halodurans* M29, generates a thermostable alkaline pectinase. This study investigated the cloning, expression, and biochemical properties of pectinase from *B. halodurans* M29.

2. Materials and methods

2.1. Chemicals and media

All chemicals used were of analytical grade. Dinitrosalicylic acid (DNSA), pectin, polygalacturonic acid (PGA), and *d*-galacturonic acid were supplied by Sigma. Several media were used to isolate the microorganisms that produce the thermostable alkaline pectinase. The contents of the various media at pH 10.0 were as follows: enriched medium: 2% pectin, 0.3% yeast extract, 0.3% K_2HPO_4, 0.02%

* Corresponding author. Tel.: +86 25 85891523.
E-mail address: meiyanzhen@njnu.edu.cn (Y. Mei).

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$MgSO4{\cdot}7H_2O$, and 1% Na_2CO_3; selecting medium (per liter): 2% pectin, 0.3% peptone, 0.03% yeast extract, 0.3% NaCl, 1% Na_2CO_3, and 2% agar; and fermentation medium: 0.5% pectin, 0.3% yeast extract, 0.3% K_2HPO4, 0.02% $MgSO_4{\cdot}7H_2O$, and 1% Na_2CO_3. The Na_2CO_3 content in all media was sterilized separately.

2.2. Microorganism, culture, and inoculum preparation

The strains were selectively enriched from soil samples based on their ability to grow enriched in the medium using the pectin the sole carbon source at 60 °C for 3–4 d. The strains were then inoculated to the selecting medium. The colonies with clear zones formed by the hydrolysis of pectin were evaluated as alkaline pectinase producers. According to the zone diameter and clearance, isolate M29 was selected as a good alkaline pectinase producer and used in all further investigations.

2.3. Strain identification based on 16S rDNA sequence and Biolog test

Bacterial 16S rRNA was amplified by polymerase chain reaction (PCR) using the primers BSF8/20 (AGAGTTTGATCCTGGCTCAG) and BSR1541/20 (AAGGAGGTGATCCAGCCGCA) [14] and sequenced by Invitrogen Corporation (Shanghai, China). The nucleotide sequence was submitted to the EMBL/Genbank/DDBJ databases under the accession number GU396007. Biolog test for the microbe was perpformed according to the manufacture's instructions (Version DE, Biolog, Harward, CA, USA).

2.4. Cloning of the alkaline pectinase gene and vector construction

Genomic DNA isolation and molecular cloning were performed following the methods described in Ref. [15]. The partial pectinase was cloned by degenerate PCR. The degenerate primers P1-1, 5′-CT(N)GG(N)GT(N)GG(N)AC(N)AA(T/C)GG-3′ and P1-2, 5′-TT(T/C)TA(T/C) AA(T/C)CA(T/C) CA(T/C) TT(T/C)AC-3′ were designed according to the amino acid alignment of pectinase [*B. halodurans* (AAW84086.1), *Bacillus clausii* KSM-K16 (YP173567.1), *Bacillus pumilus* (ZP03055090.1), and *Bacillus licheniformis* (BAL45988.1)]. The primers were used to amplify portions of the pectinase according to the following procedure: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. For genomic walking, the flanking fragments of pectinases were amplified according to the restriction site-dependent (RSD)-PCR method by Mei [16]. All PCR reactions were performed according to operational instructions. The degenerate PCR and RSD-PCR products were cloned into a thymine–adenine cloning vector according to the manufacturer's instructions. Based on the DNA sequence, the pectinase gene was amplified using primers with *Nde*I and *Hind*III restriction sites (indicated in bold): 5′-CTT**CATATG**ATGATGAGATCAAGCATC-3′ and 5′-ACA**AAGCTT**TTAAGCAATCGTTATCTTAC-3′. Thirty cycles of PCR were conducted with Pyrobest DNA polymerase (TaKaRa, P.R. China) using a 50 mL reaction mixture. Each cycle consisted of heating at 94 °C for 40 s, 51 °C for 40 s, and 72 °C for 5 min. The PCR products were purified, digested with NdeI and HindIII, and then isolated on an agarose gel. The double-digested PCR products were ligated to NdeI/HindIII-digested pET-20b (+) (Novagen, Darmstadt, Germany), and the ligation mixture was transformed into JM109 (DE3). DNA transformation was performed by electroporation. Plasmid DNA and PCR products were purified using the Qiagen plasmid and PCR purification kits (Qiagen, USA). The recombinant plasmid was isolated and confirmed by DNA sequencing using an ABI Prism 377 automated DNA sequencer. The nucleotide sequence of pectinase was submitted to the EMBL/Genbank/DDBJ databases under the accession number KC802241.

2.5. Pectinase expression and purification

Escherichia coli JM109 (DE3) cells were transformed with the recombinant plasmid and induced to express the Tma DNA ligase by adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM with optical density of approximately 0.8 at 600 nm. The cells were harvested by centrifugation (6000 × *g* for 10 min at 4 °C) washed once with deionized water, and resuspended in a small volume of 20 mM Tris–HCl buffer (pH 7.9) containing 5 mM imidazole and 0.5 M NaCl. Cell disruption was conducted by French press (11,000 psi, 2×). Cellular debris was removed by centrifugation (20,000 × *g*, 30 min, 4 °C). The suspension was heated at 80 °C for 30 min. Then, the suspension was cooled in an ice bath and centrifuged for 30 min at 20,000 × *g* and 4 °C. The supernatant was loaded on to an immobilized metal affinity column (Novagen, Inc.) and eluted with 1 M imidazole and 0.5 M NaCl in 20 mM Tris–HCl buffer (pH 10.0). The pooled fractions were dialyzed into storage buffer containing 50 mM Tris–HCl (pH 10.0), 1 mM ethylenediaminetetraacetic acid (EDTA), and 20% (v/v) glycerol before storage of the enzyme at −20 °C. The protein concentration was determined by Bradford assay. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of the purified enzyme was performed according to standard procedure.

2.6. Determination of alkaline pectinase activity and properties

The bacterial cells were pelleted by centrifugation at 9167 × *g* at 4 °C for 20 min. Alkaline pectinase in the cell-free culture filtrate was assayed by measuring the amount of *d*-galacturonic acid liberated from PGA. The reaction mixture containing 0.5 mL of appropriately diluted enzyme and 0.5 mL of 0.5% PGA (pH 10) was incubated for 5 min at 80 °C, and the end products were quantitated using DNSA reagent [17]. One unit of pectinase was defined as the amount of enzyme required to liberate 1 μmol of *d*-galacturonic acid mL^{-1} min^{-1} under assay conditions. The optimum pH for pectinase activity was determined at 80 °C for 10 min in Tris/glycine with pH values from 8.0 to 11.5. The optimum temperature for the enzymatic activity was determined from 60 °C to 95 °C in Tris/glycine (pH 10). Results were expressed as percentages of the activity obtained at either the optimum pH or temperature.

To determine the temperature stability of pectinase, the purified enzyme (0.025 μg) was pre-incubated in Tris/glycine (pH 10) at 60, 65, 70, 75, 80, 85, and 90 °C in the absence of a substrate. The pH stability of the enzyme was determined by measuring the remaining activity after the enzyme (0.025 μg) incubation at 70 °C for 1 h in Tris/glycine (pH 9.5–11). The enzymatic activity without pre-incubation was denoted as 100%.

The effects of metals on the activity of the purified enzyme (0.025 μg) were determined in the presence of 1 mM Ca^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Ni^{2+}, Mg^{2+}, Ni^{2+}, or Hg^{2+} in the reaction mixture. The effects of EDTA, SDS, and Triton-X100 on the enzymatic activity were assayed at 10 mM, 0.1% (w/v), and 0.05% (w/v), respectively. The enzyme was incubated with each reagent for 10 min at 70 °C before adding PGA to initiate the enzyme reaction. Activity was determined as described earlier and expressed as a percentage of the activity obtained in the absence of the metal cations and chemical agents.

Enzymatic determination of kinetic parameters was conducted using 20 mmol/L PGA as substrate. The enzyme was added to the substrate at 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 μL. The final volume of the enzyme reaction system was 200 μL. Enzymatic activity assay was conducted at optimum reaction conditions. The

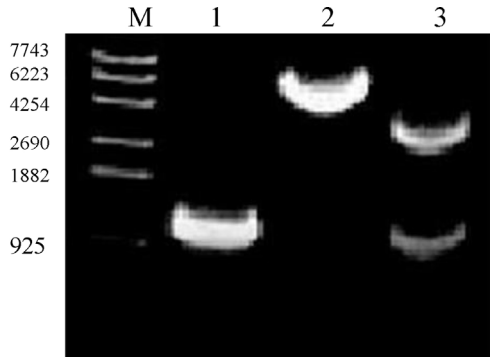

Fig. 1. PCR amplification of pectinase and enzyme identification of the recombinant plasmid.

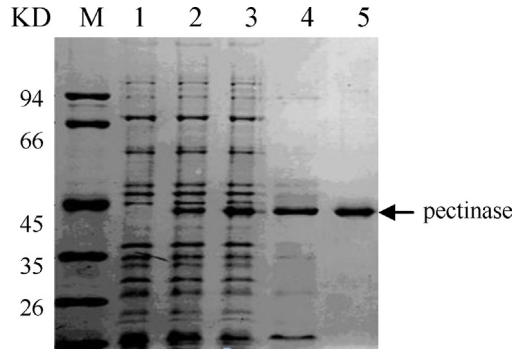

Fig. 2. SDS-PAGE analysis for the recombinant pectinase in *E. coli*.

Eadie–Hofstee mapping method was used to calculate the kinetic parameters.

3. Results and discussion

3.1. Screening and identification of alkaline pectinase-producing strains

The screening tests generated 128 strains than can grow in the selecting medium at 65 °C. The most efficient producer, strain M29, was selected based on the transparent circle generated around it in the medium containing pectin as sole carbon source (data not shown) and used in further experiments. Pectinase activity was found to be cell-associated. The strain M29 was aerobic, spore-forming, Gram-positive rod, and motile with peritrichous flagella. The strain was identified by 16 S rRNA (similarity of 98.64% with *B. halodurans* was obtained by performing a FASTA search) and Biolog test (similarity value of 0.71 for *Bacillus* was obtained by comparing with the database for microbe in Biolog). Thus, strain M29 was identified as a strain of *B. halodurans*.

3.2. Pectinase cloning and plasmid construction

The whole pectinase gene sequence was obtained by degenerate PCR and genomic walking according to a previously reported method [16]. The gene encoding pectinase was amplified from the genomic DNA of *B. halodurans* M29 and inserted into the gene expression vector pET-20b (+) at the *Nde*I and *Hind*III sites (Fig. 1). The pectinase gene encoded 352 amino acid with a molecular mass of 39 Da and calculated p*I* of 6.19. The protein included a typical amino-terminal signal sequence at positions 31 and 32 of the protein sequence, which was identified by the program SignalP [18].

3.3. Alkaline pectinase gene expression and purification

The recombinant plasmid was designated as pET-pectinase, which was transformed into *E. coli* JM109 (DE3) for pectinase production. Pectinase was expressed with a C-terminal His-tag. Most of the *E. coli* proteins were eliminated effectively after heat treatment at 70 °C for 30 min. After heat treatment and affinity chromatography, the recombinant enzyme in the cell-free extracts was purified to gel electrophoretic homogeneity (Fig. 2). The yield of the purified protein was 1.23 mg L^{-1}. SDS-PAGE showed that the recombinant pectinase had a molecular mass of approximately 44 kDa. As shown in Table 1, most of the heterozygous protein and pigment were removed by heat treatment, and the purification factor increased to 4.5. Enzymatic activity recovery after purification with DEAE-Sepharose Fast Flow (FF) was 24.8%, and the purification increased by 10.9-fold. Specific activity increased from 13 U mg^{-1} to 142 U mg^{-1}. The dependence of the enzyme reaction rate on the PGA concentration followed the Michaelis–Menten kinetics, with apparent K_m and V_{max} values of 4.1 g L^{-1} mM and 351 U mg^{-1} protein, respectively. The kinetic constants of pectinase were determined by measuring the initial rates at various PGA concentrations under standard reaction conditions.

3.4. Effects of pH and temperature on enzyme activity and stability

When PGA was used as the substrate, the optimal reaction of alkaline pectinase occurred at 80 °C and pH 10 (Fig. 3A and B). The purified enzyme retained over 60% of its activity from pH 8 to 11 for 1 h and 80 °C (Fig. 3C). Thermostability was evaluated by determining the residual activity after incubating the mixtures (0.2 mg mL^{-1} pectinase, 50 mM Tris–HCl, pH 10.0) for various durations at 70, 75, 80, and 85 °C. The residual activity under standard reaction conditions is demonstrated in Fig. 3D. After incubation at 70 °C, the alkaline pectinase retained its activity, implying that no irreversible unfolding of the protein occurred. The enzyme had a half-life of approximately 90 min at 80 °C, indicating that it could stand the temperature cycles of PCR.

3.5. Required cofactor and divalent cation for pectinase activity

The purified enzyme was incubated with various metal ions (1 mM) or chemical reagents at 80 °C for 30 min, and then residual activities were assayed by standard method. As shown in Table 2, the pectinase activity was absolute requirement for Ca^{2+}, partly inhibited by Mg^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Mg^{2+}, Ni^{2+}, or Hg^{2+}, and completely inhibited by Co^{2+}. This result is different from those of other previously reported pectinases, in which pectinase activities were inhibited rather than increased by Ca^{2+}. The difference may have been caused by the amino acid sequence of pectinase. Pectinase was sensitive to 10 mM (EDTA), 0.1% (w/v) SDS, and 0.05%

Table 1
Purification process of alkaline pectinase in *E. coli* JM109 (DE3).

Step	Total protein (mg)	Enzyme activities (U)	Specific activity (U/mg)	Recovery	Purification fold
Crude extraction	287	3731	13	100	1
Heating	23.1	1359	58.8	36.4	4.5
DEAE-Sepharose FF	6.5	926	142	24.8	10.9

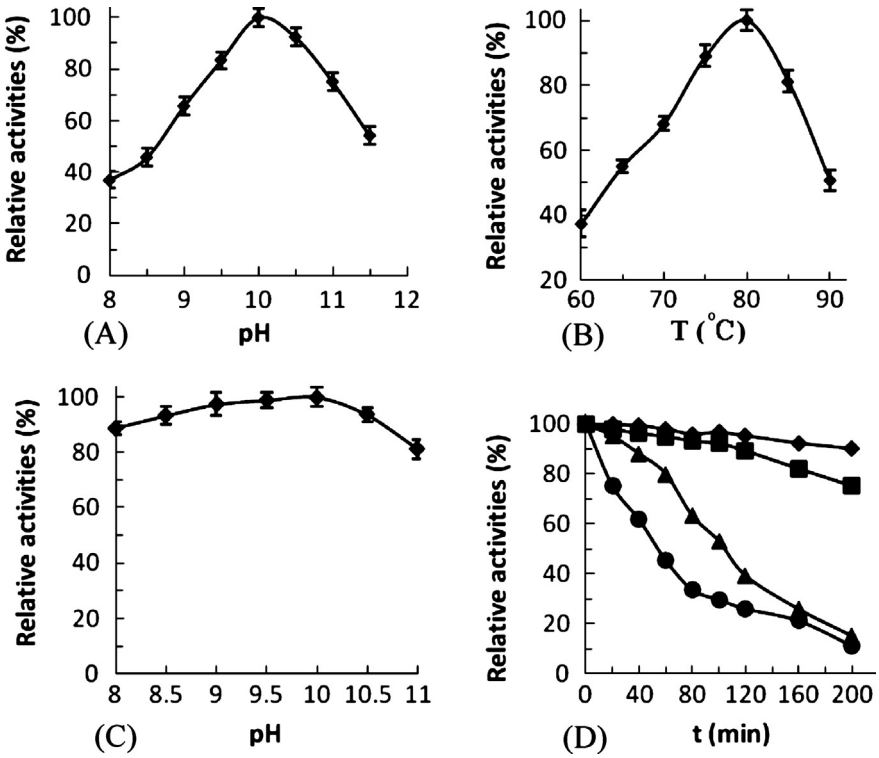

Fig. 3. Effects of pH and temperature on the activity and thermostability of pectinase.

Triton-X100, as shown by the 20.7%, 32.4%, and 15.8% activities retained, respectively.

4. Discussion

Alkaline pectinase, which is produced by various microorganisms, is applied in various industrial applications [19]. The pectinase of *B. halodurans* M29 isolated from a hot spring had high thermostability and showed good potential. The pectinase was heterologously expressed and characterized the pectinase from *Bacillus halodurans* M29, which displays original features such as high activity and thermostability. The pectinase from *B. halodurans* M29 was optimally active at pH 10.0 and 80 °C. Although a few thermotolerant pectinases have already been reported, their threshold temperature was lower than that of the pectinase from M29. The pectinase of *Bacillus pumilus* dcsr1 is optimally active at pH 10.5 and 50 °C [3]. Two pectate lyase genes from the pine wood nematode *Bursaphelenchus xylophilus* have optimal activity at pH 9.0 and 55 °C [20]. The polygalacturonate lyase of *B. pumilus* is optimally active from pH 8.0 to 8.5 and at 60 °C [21]. The pectate lyase from *B. pumilus* BK2 exhibits optimum activity at pH 8.5 and approximately 70 °C [22]. The enzyme of *B. pumilus* DKS1 exhibits maximal activity at pH 8.5 and 75 °C [23]. The enzyme of *Bacillus* sp. RN1 exhibits maximal activity at pH 10 and 90 °C [24]. The pectinase of *B. halodurans* M29 had high thermostability at 70 °C, and the half-life was 90 min at 80 °C. The pectate lyase of *B. pumilus* BK2 exhibits a half-life of 75 h at 30 °C [22]. The pectate lyase of *B. pumilus* DKS1 has a half-life of 3.75 min at 80 °C [23]. The enzyme of *Bacillus* sp. RN1 has a half-life at of 60 min at 55 °C [24]. The pectate lyase of M29 showed maximal activity at a high temperature and has a long half-life. By contrast, most pectate lyases are active at lower temperatures or have shorter half-lives. Therefore, compared with the pectate lyases of other isolated strains, the pectinase of M29 was thermostable.

Table 2
Effect of metal ions and inhibitors on the activity of the recombinant pectinase.

Metal ions and surfactants	Pectate lyase C (relative activity, %)
Ca^{2+}	100
Mn^{2+}	36.8
Co^{2+}	0
Cu^{2+}	21.3
Mg^{2+}	76.7
Zn^{2+}	29.6
Ni^{2+}	14.7
Hg^{2+}	4.6
EDTA	32.4
SDS	20.7
Triton-X100	15.8

The M29 strain had an absolute requirement for Ca^{2+} to stimulate its enzymatic activity and requires an alkaline pH for optimum activity. Similar results have been observed with *B. pumilus* DKS1 [23], *B. pumilus* [21], *Bacillus polymyxa* [25], *and B. xylophilus* [20,24]. However, the pectate lyase from *B. pumilus* BK2 [19] does not require Ca^{2+}. This finding is also confirmed by the loss of activity of the M29 enzyme in the presence of EDTA. Co^{2+} is a potent inhibitor of the pectate lyase from *B. halodurans* M29. Similar results have been observed with *B. pumilus* BK2. The pectate lyase from *B. pumilus* DKS1 lost only 6% of its total activity in the presence of Co^{2+}. Thus, the pectate lyase from *B. halodurans* M29 exhibits unique characteristics in terms of its activity in the presence of metal ions compared with the enzyme from a separate *B. pumilus* BK2 strain.

The amino acid sequence of the pectinase reported in this study was compared with five other pectinases. The amino acid sequence identity to the pectinase of *B. halodurans* (AAW84086.1), *Thermotoga maritima* (ZP12684458.1), *B. clausii* KSM-K16 (YP173567.1), *B. pumilus* (ZP03055090.1), *Paenibacillus mucilaginosus* 3016 (YP_005311158.1), and *B. licheniformis* (BAL45988.1) was 55%, 54%, 40%, 40%, 36%, and 31%, respectively. High amino acid sequence homology was observed between the pectinases from other thermophilic microorganisms such as *Bacillus* sp. However, the pectinase from the strain M29 showed low amino acid sequence

homology with the enzymes from mesophilic microorganisms such as *B. pumilus* (ZP03055090.1) and *B. licheniformis* (BAL45988.1). These results imply that the pectinases from thermophiles are closely related to each other. These characteristics suggest the evolutionary diversity of pectinase.

Moreover, the pectinase of *B. halodurans* M29 could be easily purified, using only heat treatment and DEAE-Sepharose FF, when it was expressed in *E. coli* because of its thermostability at high temperature. Conversely, other proteins lose their activity at the same temperature. From these results, we concluded that pectinase from the M29 strain is a novel alkaline enzyme (only 54% sequence similarity to known enzymes). Given its high thermostability and long half-life, this pectinase can be potentially applied in industrial processes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.05.004.

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